A laboratory simulation of Arabidopsis seed dormancy cycling provides new insight into its regulation by clock genes and the dormancy-related genes *DOG1*, *MFT*, *CIPK23* and *PHYA*

Running title: Dormancy cycling with mutants

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Abstract

Environmental signals drive seed dormancy cycling in the soil to synchronise germination with the optimal time of year; a process essential for species fitness and survival. Previous correlation of transcription profiles in exhumed seeds with annual environmental signals revealed the coordination of dormancy regulating mechanisms with the soil environment. Here, we developed a rapid and robust laboratory dormancy cycling simulation. The utility of this simulation was tested in two ways. Firstly using mutants in known dormancy-related genes (*DELAY OF GERMINATION 1* (*DOG1)*, *MOTHER OF FLOWERING TIME* (*MFT)*, *CBL-INTERACTING PROTEIN KINASE 23* (*CIPK23)* and *PHYTOCHROME A* (*PHYA)*). Secondly, using further mutants we test the hypothesis that components of the circadian clock are involved in coordination of the annual seed dormancy cycle. The rate of dormancy induction and relief differed in all lines tested. In the mutants, *dog1-2* and *mft2,* dormancy induction was reduced but not absent. *DOG1* is not absolutely required for dormancy. In *cipk23* and *phyA* dormancy induction was accelerated. Involvement of the clock in dormancy cycling was clear when mutants in the morning and evening loops of the clock were compared. Dormancy induction was faster when the morning loop was compromised and delayed when the evening loop was compromised.

Key words: *Arabidopsis*, circadian clock, circannual rhythm, DOG1, dormancy cycling, germination, seed dormancy, thermal time.

Introduction

Seeds are highly efficient sensors and interpreters of the prevailing environment and their environmental history. Seeds first sense the maternal environment to set the depth of primary dormancy at maturity (e.g. temperature) (Kendall *et al.* 2011; Penfield and Springthorpe, 2012; He *et al.* 2014; Huang *et al.* 2014 & 2015; Chen *et al.* 2015). Seeds that do not germinate immediately upon shedding enter the soil seed bank and respond to the soil environment by continually adjusting depth of dormancy to time the eventual completion of germination (Footitt *et al.* 2011, 2013, 2014; 2015; Finch-Savage and Footitt, 2012, 2017; Penfield and Springthorpe, 2012). When depth of dormancy is low seeds are sensitive to signals that inform of the spatial environment (e.g. light, nitrate and temperature). If these signals are not received to remove the final layer of dormancy then seeds enter secondary dormancy (Finch-Savage and Footitt 2017). In this way seeds determine the time and place of plant establishment to synchronise their life cycle with favourable environments (Finch-Savage and Leubner-Metzger, 2006; Burghardt *et al.* 2016; Springthorpe and Penfield 2015). Recent correlations of annual gene expression patterns in exhumed seeds with environmental signals in the field provided the first insight into the temporal integration of the molecular regulation of dormancy cycling (Footitt *et al.* 2011, 2013, 2014).

However, studying dormancy cycling in the field is a long-term undertaking and ethical and regulatory reasons can preclude the use of seeds from genetically modified plants to dissect the role of individual genes; progress in understanding has therefore been slow. To address this in the work presented we used our field and laboratory observations (Cadman *et al.*

2006; Footitt *et al.* 2011; Huang *et al.* 2015) to develop a representative, but rapid and simple laboratory simulation of dormancy cycling. It enables a full dormancy cycle to be completed in *c*. eight weeks. The method was developed using the deeply dormant winter annual ecotype Cvi then applied to the Col-0 and Ler ecotypes to facilitate mutant analyses. Here we explore the utility of this laboratory simulation in two ways.

Firstly, we use the simulation to confirm the involvement of genes previously identified in correlative gene expression studies as central to the regulation of seed dormancy cycling (Footitt et al, 2011, 2013). These studies suggest that by influencing the central integrating hormonal balance (Abscisic acid (ABA)/Gibberellins (GA)) DELAY OF GERMINATION 1 (DOG1) and MOTHER OF FLOWERING TIME (MFT) play key roles in the response to temporal signals (e.g. temperature) that regulate dormancy cycling (Finch-Savage and Footitt, 2017). This is linked to temporal changes in the expression of PHYTOCHROME A (PHYA) and CBL-INTERACTING PROTEIN KINASE 23 (CIPK23) that alter sensitivity to signals indicating suitability for germination completion (spatial signals: light and nitrate respectively). Once sensitised, seeds respond to these signals through the ABA/GA balance to bring about the completion of germination when conditions are optimal (Finch-Savage and Footitt, 2017). We confirm the involvement of these genes in ABA sensitivity and dormancy cycling using mutant lines (*dog1-2*, *mft2*, *cipk23* and *phyA*). In the field, dormancy induction and relief during cycling were shown to progress in thermal time (Footitt *et al*. 2011) and we use this approach to analyse data in the present work. Thermal time is quantified as the amount by which temperature exceeds a minimum temperature or threshold for the process in question. When this value is summed over days to give degree days (°C days) thermal time

can be used to measure progress towards the completion of that process (Finch-Savage and Luebner-Metzger, 2006).

Secondly, we use the simulation to test the previously unstudied hypothesis that components of the circadian clock may be involved in coordination of the annual seed dormancy cycle. The regulation of daily circadian rhythms has been extensively studied in plants (e.g. Salome and McClung 2005; Seung *et al*. 2012; Seo and Mas 2015; and Atamian and Harmer 2016). In a 24 hour cycle, interlocking morning and evening feedback loops control the period and phases of the circadian clock. The morning loop in Arabidopsis contains the MYB-related transcription factors *LATE ELONGATED HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) whose increased expression represses the evening loop gene *TIMING OF CAB EXPRESSION* (*TOC1*). LHY/CCA1 also induce the sequential expression of the *PSEUDO RESPONSE REGULATOR* genes *PRR9, PRR7,* and *PRR5*; which feedback during the day to progressively repress *LHY/CCA1* and therefore relieve repression of *TOC1*. The latter protein, which induces *LHY/CCA1* expression (as reviewed in Hsu and Harmer, 2014) is targeted for degradation by GIGANTEA (GI) in conjunction with ZEITLUPE (ZTL). GI then appears to be repressed by EARLY FLOWERING 3 (ELF3) a member of the evening complex (Mishra and Panigrahi, 2015). The evening complex is formed by the proteins ELF3, ELF4 and LUX ARRYTHMO (LUX) and this represses the expression of the day-phased clock gene *PRR9* (Hsu and Harmer, 2014).

Some of these genes are known to influence seed dormancy. For example, *LHY* and *CCA1* mutants were more sensitive to dormancy relieving low temperature stratification and the *GI* mutant less sensitive (Penfield and Hall, 2009). They also alter the hormone balance in

seeds. For example, *GI* and *TOC1* mutants influence ABA and GA sensitivity and the expression of ABA and GA related genes (Penfield and Hall, 2009). The clock was also a key regulator of physiological activity when dormancy of imbibed *Euphorbia esula* seeds was relieved by alternating temperature in the dark (Foley *et al.* 2010). This indicates that the clock could respond to temperature signals in the dark conditions experienced in the soil seedbank; furthermore temperature alternations of 4°C are known to entrain the clock (Salome and McClung, 2005). However, in seeds it is the rhythm of the seasonal temperature cycle that provides a temporal signal indicating time of year (Probert, 2000). In other tissues, components of the circadian clock can respond to seasonal changes in day length and associated alternation of light and temperature to coordinate tree bud dormancy with the time of year (Cooke *et al.* 2012). A similar role for the clock in seeds is largely unexplored. To address this we use a targeted selection of mutant lines of clock genes to determine their contribution to the dormancy cycle and associated ABA sensitivity. We complement this by analysing the annual transcription profiles of these genes during dormancy cycling in the field.

Materials and Methods

Seed production: Seeds of the Arabidopsis Cape Verde Island (Cvi) and Burren (Bur) ecotypes were produced in a heated glasshouse with supplemental lighting in 2007 (Cvi) and 2008 (Bur) and were harvested, processed and then stored at -80°C as described elsewhere (Footitt *et al*. 2011, 2013). Seeds of the Arabidopsis mutants' *toc1-101* (Kikis *et al.* 2005), *lhy20 cca1-1* (Yakir *et al.* 2009), *lhy20 cca1-1 toc1-2* (Yamashino *et al.* 2008), *prr5-11 prr7-11 prr9-10* (Nakamichi *et al.* 2005), *dog1-2* (Nakabayashi *et al.* 2012), *mft2* (Xi *et al.* 2010),

cipk23 (N503652) and *phyA* (N6223) are in the Col-0 (N1092) genetic background. While the overexpressing lines *LHY-OX* and *CCA1-OX* are in the Ler and Col-0 backgrounds respectively (Green *et al.* 2002). All lines and their wild types were produced in the same growth cabinet (16°/16°C 16h L/8 h D). Following harvesting and processing seeds were stored at -80°C (See supplementary methods for seed production conditions).

Dormancy cycling in the laboratory

The annual variation in soil temperature and water potential are seen to impact the annual seed dormancy cycle in the field (Footitt *et al.* 2011). These observations were used to develop a protocol for dormancy cycling in the laboratory. Dormancy/germination experimental treatments and procedures used surface sterilised seeds and were all carried out in the dark under a green safe light unless otherwise stated.

Impact of water potential on dormancy status in Cvi: Decreasing soil water potential was associated with low temperature induction of dormancy in Cvi in the field (Footitt *et al*. 2011). Consequently its role was tested in the laboratory. Dormant seeds were surface sterilised in 2.5% dilution of domestic bleach for 5 minutes and washed three times in water. Seeds were then placed (3 x 40 seeds) into boxes (124 x 88 x 22 mm) (Stewart Plastics Ltd, UK). Each box contained 25 ml of solution set at a range of water potentials (0, -0.4, -0.8, and 1.2 MPa) using PEG 8000. This PEG solution volume represents a solution volume/paper weight ratio of 3.55 that minimises the concentrating effect of filter paper on the solution (Hardegree and Emmerich 1990). This liquid reservoir was accommodated beneath the seeds as follows. In the base of each box was placed a piece of glass-drying mat (Nisbits Ltd, UK). The drying mat was an open lattice 3 mm deep to create space for the PEG

156 solution. On top of this was placed nylon mesh (1 mm mesh size) (Plastok, UK) to support
157 the single sheet of Whatman 3MM chromatography paper (Camlab, UK) that is then placed
158 on top. Strips of nylon mesh (125 µm mesh size, 45% open mesh) (Plastok, UK) were then
159 laid on the paper and each seed replicate was placed on one of those individual strips (for a
160 visual representation see Fig. S1). Boxes were then sealed inside freezer bags to minimize
161 evaporation and wrapped in two layers of aluminium foil to exclude light and incubated at
162 5°C for up to 14 days. Germination tests were carried out on these seeds after increasing
163 intervals at 5°C. The nylon strips holding the seeds were transferred to new boxes
164 containing 2 sheets of chromatography paper and 8 ml of 50 or 250 µM Gibberellin $_{4+7}$ in
165 citrate/phosphate buffer (pH 5.0) or a buffer control in the light at 20°C and germination
166 recorded over 28 days (Footitt *et al.* 2011). Gibberellin $_{4+7}$ was dissolved in 100 µl 0.1 M KOH
167 before preparing stock solution.

168 Seeds incubated on water (0 MPa) were also transferred at intervals to fresh water, or 10
169 mM KNO_3 and incubated at 20°C/light for 28 days to record germination. In all treatments
170 dark germinated seeds were recorded on transfer to the light. Germination was recorded as
171 protrusion of the radicle through the seed coat and micropylar endosperm.

172 **Dormancy cycling in Cvi:** The constrasting impact of winter and summer temperature on the
173 annual dormancy cycle of Cvi was simulated using lower and higher constant temperatures
174 to simulate dormancy cycling in the laboratory. Dormant seeds were plated (3 x 40 seeds)
175 onto nylon mesh strips in boxes containing a -1.2 MPa PEG 8000 solution as above and
176 incubated at 5°C for up to 21 days. At this point seeds were transferred to boxes containing
177 2 sheets of chromatography paper and 8 ml water and incubated at 25°C for 35 days. At
178 each transfer point dark germinated seeds were counted. At intervals, boxes were removed

and dormancy tested by transferring seeds to boxes in the light as above containing 50 or 250 µM Gibberellin 4+7 or a buffer control as above.

Dormancy cycling in Col-0 wild type and mutants: Using the laboratory dormancy cycling simulation, mutants in the Col-0 genetic background were used to test the contribution of selected genes to dormancy cycling. Seeds were plated (3 x 40 seeds) into boxes containing a -1.0 MPa PEG 8000 solution as above. A series of experiments testing a wide range of treatment temperatures and durations were then performed to evaluate the role of temperature in the induction and relief of dormancy. For the impact of cold conditioning on high temperature dormancy induction seeds were incubated at 5°C/ -1 MPa for up to 28 days then transferred to germination plates containing 2 sheets of chromatography paper and 8 ml of water and incubated at 20 (clock mutants only), 25 and 30°C for up to 14 days at which point plates were transferred to 5°C for up to 29 days. At intervals during each incubation period boxes were removed for germination testing at 25°C/light for 14 days. Loss of dormancy in the presence of nitrate in Col-0 and *cipk23* seeds subjected to 5°C/ -1 MPa followed by 25°C was also tested by transferring seeds to 10 mM KNO3 at 25°C/light for 14 days. Germination tests were carried out at 25°C as the Col-0 wild type retains greater thermodormancy at this temperature compared to 20°C. Seeds on PEG 8000 solution were transferred to water prior to germination testing in the light. Dark germination was recorded at each transfer point and prior to germination testing.

ABA sensitivity: As changing ABA sensitivty has in integral role in the dormancy continuum the sensitivity of mutants was determined. Seeds were plated on to nylon mesh in boxes containing water as above. They were cold stratified at 5°C/dark for three days then transferred to boxes containing 10 - 250 nM (±)-ABA (Sigma, UK) in citrate/phosphate buffer

(pH 5.0) and incubated in the light at 25°C. ABA was dissolved in 100 µl 0.1 M KOH before preparing stock solution.

Dormancy cycling in the field and gene expression analysis: As seed dormancy cycling displays an annual rhythm in response to seasonal soil temperature patterns we determined the transcriptional profile of seven clock genes in seeds recovered over twelve months from field soil. Experiments on dormancy cycling in the field were performed as described previously (Footitt *et al.* 2011 and 2013). Seeds were recovered from the soil in the morning of the day of harvest. QPCR of circadian clock gene expression was performed using the touchdown PCR thermal cycle: one cycle at 95°C for 10 min followed by 50 cycles at 95 °C for 30s, 70°C (decreasing by 0.2°C/cycle to a target temperature of 67°C) for 30s, and 72 C for 30s. All other details regarding QPCR procedures and analysis were described previously (Footitt *et al.* 2015). Primer sequences are given in Table S2.

Results

Dormancy cycling in Cvi under laboratory conditions:

Cvi seeds in field soil are induced into deeper primary dormancy by low temperatures in winter, dormancy then declines to low levels in response to higher temperatures in spring/summer; deeper dormancy (secondary dormancy) is then re-induced by autumn/winter low temperatures (Footitt *et al*. 2011). A series of experiments were conducted to reproduce this behaviour in the laboratory. Primary dormant seeds on water did not germinate in the dark and germination was less than 5% at 20°C/light (Fig. 1(a)). Periods of pre-exposure to low temperature in the dark up to 14 days had a limited effect

on germination on water (Fig. 1a) and the buffer control (Fig. 1(b)). Germination on nitrate marginally increased from 5-17 % in the first 4 days, then declined on further exposure. However, full germination was induced by imbibition on GA (250 μM GA_{4+7}), which then progressively declined on exposure to low temperature indicating an increasing depth of dormancy not evident on water, or the buffer control (Fig. 1b). Depth of dormancy increased more rapidly on exposure to low temperature when seeds were incubated in negative water potentials (down to –1.2 MPa) consistent with the observations of Auge *et al.* (2015).

A second lot of seeds from the same harvest were exposed to low temperature (5°C/dark) for 21 days with and without water stress at -1.2 MPA and then transfered to water at 25°C/dark to simulate a full dormancy cycle (Fig. 2). This second seed lot had been stored at -20°C, which resulted in a lower dormancy level. With these seeds, germination on the buffer control increased to < 40% after 6 days of low temperature indicating this proportion of the population had the lowest level of primary dormancy. In this portion, dormany could then be relieved by light, with the remainder not yet light sensitive. With continued low temperature exposure deeper dormancy was induced in the population as a whole. Sensitivity to GA_{4+7} declined (i.e. dormancy deepened) so that no seeds germinated even at 250 μM GA_{4+7} after exposure to low temperature for 21 days. Depth of dormancy then declined progressively in the subsequent high temperature phase of the cycle. This began after 2 days on GA and then later in the control after 40 days.

Dormancy cycling in Col-0 and Ler:

Col-0 and Ler seeds were produced by maturing them at the relatively low temperature of 16°C. This lower temperature increased the level of primary dormancy and prevented dark germination at low temperture. Subsequent imbibition of these seeds at low temperature relieved primary dormancy and high temperature then induced secondary dormancy (Fig. 3). Dormancy was initially similar in Col-0 and Ler, but the exposure to low temperature revealed Ler was the more dormant ecotype (Fig 3(a) & (c)).

To simulate a dormancy cycle in the laboratory, Col-0 and Ler seeds were first subjected to low temperature (5°C/dark) at -1.0 MPa for up to 28 days. On day 28, seeds were transferred to water at 25 °C/dark (Fig. 3; results at 25 and 30 °C are shown in Fig. S2, also 20, 25 and 30 °C in Fig S3, & S4), which resulted in the rapid induction of secondary dormancy in all seeds. This was followed by a second low temperature phase to relieve secondary dormancy. At all stages dormancy level was determined by germination following transfer to 25°C/light. At this temperature these ecotypes exhibit high temperature thermodormancy. Seeds from lines with mutated dormancy regulating genes (*dog1-2*, *mft2*, *cipk23* and *phyA*) and mutated clock genes and over expressing lines were also subjected to this simulation.

To test if incubation of Col-0 seeds beyond 28 days at low temperature (5°C/dark) at -1.0 MPa would induce secondary dormancy seeds were incubated for up to 42 days. Although primary dormancy was relieved and germination was 94% after 21 days it only declined to 84% after 28 days and 83% at 42 days indicating a slow induction of secondary dormancy that may increase if the treatment was extended further(see Discussion).

Dormancy cycling in mutants of dormancy-related genes: Primary dormancy in the mutants *mft2*, *cipk23* and *phyA* was similar to Col-0, but *dog1-2* was non-dormant (Day 0; Fig. 3(a)). The response of these mutants to the dormancy cycle simulation differed greatly from Col-0. The initial low temperature phase relieved primary dormancy and then induced secondary dormancy in *cipk23* and *phyA* after 21 days. On transfer to higher temperature (25°C), secondary dormancy induction was complete after only four days in *cipk23* and *phyA*, but seven days in Col-0 (Fig. 3(a)). In contrast, *dog1-2* and *mft2* secondary dormancy induction was slower. Maximum induction was after 14 days in *dog1-2* (germination 48%) and seven days in *mft2* (germination 33%). On transfer to the second low temperature phase, secondary dormancy was broken after two days in *dog1-2* and *mft2;* and after four days in Col-0, *cipk23* (83%) and *phyA* (65%). Secondary dormancy was then re-induced in Col-0, *cipk23* and *phyA*, but not in *dog1-2* and *mft2*. The rate of change was greater when 30°C was used to induce secondary dormancy, but the relative performance of the lines was very similar (Fig. S2). As CIPK23 is involved in the regulation of nitrate transport and signalling the nitrate sensitivity of Col-0 and *cipk23* was tested when secondary dormancy was induced at 25°C for 14 days. Germination was 85% and 77% respectively in the presence of 10mM nitrate at 25°C/light.

To determine the role of the initial cold treatment seeds were exposed directly to high temperature (25 or 30°C) in the dark. Secondary dormancy was not induced in *dog1-2* but was in the wildtype and the other dormancy related mutants (Fig. S5).

Selection of lines to test whether clock genes influence seed dormancy cycling: We subjected seeds from lines with the following clock mutations: *toc1-101*, *lhy20 cca1-1*, *lhy20*

296 *cca1-1 toc1-2* and *prr5-11 prr7-11 prr9-10* and the overexpressing lines *LHY-OX* and *CCA1-*
297 *OX* to the dormancy cycling simulation. This combination of mutants allowed us to
298 investigate whether altering the balance between the morning and evening loops of the
299 clock would alter the dormancy cycling response under the relatively long-term, but
300 changing, constant temperatures of the simulation in the dark (i.e. in the absence of an
301 imposed external daily rhythm).
302
303 **Dormancy cycling in clock mutant lines:** Primary dormancy of all lines was initially relieved
304 during the low temperature phase, but secondary dormancy induction started between
305 days 21 and 28 except in *prr5-11 prr7-11 prr9-10*; and induction increased in the order *toc1-*
306 *101*, *lhy20 cca1-1*, and *lhy20 cca1-1 toc1-2* (Fig. 3(b)). On transfer to higher temperature
307 (25°C) at 28 days, secondary dormancy was completely induced after a further seven days
308 and was slowest in *prr5-11 prr7-11 prr9-10* . On transfer back to low temperature secondary
309 dormancy was rapidly relieved and then re-induced in all lines (Fig. 3(b)). The impact of the
310 high temperature phase on rate of dormancy induction and its subsequent relief differed
311 with temperature (20, 25, & 30°C, Fig. S3)
312
313 The overexpressing lines behaved differently from their respective wild type comparisons. In
314 the *CCA1-OX* (Col-0 background) overexpressing line secondary dormancy was more rapidly
315 induced during the first low temperature phase than in Col-0 and was complete after only
316 four days on transfer to 25°C compared to 7 in Col-0 (Fig. 3(c)). Whereas, the *LHY-OX* (Ler
317 background) overexpressing line was more dormant than Ler and the first low temperature
318 phase did not relieve dormancy. Dormancy increased on transfer to 25°C (Fig. 3(c)). In the
319 second low temperature phase, secondary dormancy was relieved, but rapidly re-induced

only in *CCA1-OX*. The response to the second low temperature phase was dependent on the previous temperature. For example, in contrast to that shown on transfer from 25°C (Fig. 3(b) & S4(b)), on transfer from 20°C secondary dormancy was not relieved by low temperature in *CCA1-OX* (Fig. S4(a)). Furthermore, on transfer from 30°C to low temperature secondary dormancy was relieved, but only re-induced with prolonged incubation (Fig. S4(c)).

Response of clock genes to temporal signals in the field: To further understand the response of clock mutants we analysed the transcription profiles of selected clock genes in seeds of the deeply dormant winter annual ecotype Cvi and the shallow dormant summer annual ecotype Bur during dormancy cycling in the field (Fig. 4). Bur is a summer annual ecotype whose dormancy cycling behaviour is highly characterised (Footitt *et al.* 2013; 2015). As such it is used here as a model for the summer annual behaviour of the ecotype Col-0 the genetic backgound of the clock mutants used in the laboratory simulation. In both Cvi and Bur ecotypes there were clear annual transcript profiles, however the profiles of the morning genes *CCA1* and *LHY* differed between ecotypes (Fig. 4 (b) & (f)). In Cvi, the transcription profiles of *LHY* and *TOC1* where similar; but opposite to the soil temperature profile. Whereas in Bur, *CCA1* and *TOC1* transcript profiles are similar, but have little relationship with the temperature profile (see Table S1). In Cvi and Bur, *GI, PRR7* and *ELF3* transcription profiles are the same and inversely tracked soil temperature and in the case of Cvi also tracked dormancy (Fig. 4(c), (d), (g) & (h)). Of the evening complex genes examined, *LUX* transcription had no obvious pattern in contrast to *ELF3* (Fig. 4(d) & (h)).

Significant correlations occurred between the transcription-profiles of the clock and dormancy related genes and the annual soil temperature cycle in both ecotypes (Table S1). In particular there were strong correlations between the evening genes *ELF3* and *GI* and chromatin remodelling genes involved in gene activation/repression such as *HUB1* and *OTLD1* and silencing (*KYP*/*ROS1*) (Footitt *et al.* 2015) (Table S1).

Dormancy induction and thermal time: In the field, dormancy induction and relief during cycling were shown to progress in thermal time (Footitt *et al*. 2011). We therefore used this approach to analyse data in the laboratory simulation during dormancy induction at 20, 25 and 30°C (Fig. 5 & S6). Induction of secondary dormancy in Col-0, *mft2* and the clock mutants followed an exponential decay response with thermal time. In the clock mutants the thermal time required to induce secondary dormancy in 50% of the population decreased in the order *prr5 11 prr7-11 prr9-10* (153 °C days)> Col-0 (105 °C days)> *toc1-101* (82 °C days)> *lhy20 cca1-1* (60 °C days)> *lhy20 cca1-1 toc1-2* (54 °C days). In *dog1-2* induction of secondary dormancy had a linear response (see Fig. S6 for regression equations).

ABA sensitivity of Col-0, dormancy and clock mutants: Due to the role of ABA in the induction of dormancy we investigated ABA sensitivity of both groups of mutants. Dormancy mutants showed large differences in ABA sensitivity. With the exception of *dog1-2* final germination was similar in all lines (Fig. 6(a)). However, the speed of germination represented by the time to 50% germination (T50) (a measure of germination velocity) revealed that ABA sensitivity increased in the order *dog1-2* > *mft2* > *Col-0* > *phyA* > *cipk23* (Fig. 6(b)) similar to that seen for the induction of secondary dormancy in thermal time (Fig.

5(a) & S6(a)). The response of dormancy mutants to 100 nM ABA (Fig. 6(c)) illustrates further their different ABA sensitivities. ABA sensitivity in *dog1-2* was greatly reduced in agreement with that reported for *dog1-1* (Ler background) (Bentsink *et al.* 2006). The onset of low ABA sensitivity was delayed in *mft2* potentially indicating delayed ABA catabolism as an uplift in germination occured at the same time in all lines (Fig. 6(c)).

Clock mutants also exhibit different ABA sensitivities (Fig 6(d)). The time to 50% germination (T50) revealed that ABA sensitivity increased in the order *prr5 11 prr7-11 prr9-10* > *toc1-101* > Col-0 > *lhy20 cca1-1* > *lhy20 cca1-1 toc1-2* (Fig. 6(e)) again similar to secondary dormancy induction in thermal time (Fig. 5(b)). The response to 50 nM ABA indicates these differences are constant during germination (Fig. 6(f)). The overexpressing lines have similar ABA sensitivity to there parental wild types (Fig. S7).

Germination in the dark: Germination is reported following transfer to the light including the limited dark germination. Dark germination was also recorded to determine if temperature manipulation replaced dormancy relief by light. There was no dark germination in the first low temperature phase. In the high temperature phase dark germination in the dormancy mutants peaked at 25% in *dog1-2* and 32% in *mft2* at 25°C; and 9% and 2% at 30°C (Fig. S8). In the clock mutants and over expressers, dark germination was 5% or less at 20 and 25°C (Fig. S9) with none at 30°C. In Col-0, maximum dark germination at high temperature was 11% (Fig. S8 & S9). In the second low temperature phase, dark germination shows little response. In *cipk23* and *phyA*, maximum dark germination was 2%.

Discussion

Gene expression patterns during annual dormancy cycling in the field can differ from those anticipated from more static laboratory dormancy studies (Footitt *et al*, 2011, 2013; Finch-Savage and Footitt, 2017). We therefore established a robust laboratory simulation of dormancy cycling in different Arabidopsis ecotypes by manipulating temperature and water potential. The central role of temperature in dormancy cycling is well known (Probert 2000); and the role of low water potential on the induction of secondary dormancy in the dark was originally shown by Khan and Karssen (1980). Furthermore, primary dormancy status upon shedding is known to influence subsequent cycling, for example, it can impact on the induction of secondary dormancy by low water potential in Col-0 (Auge *et al*., 2015). In the experiments presented, ecotypic differences in the relief and induction of dormancy by the temperatures used in the simulation were consistent with those previously shown for Bur, Col-0, Ler, and Cvi (Cone and Spruit, 1983; Huang *et al.* 2015, Springthorpe and Penfield 2012, 2015). These differences presumably arose during adaptation to their specific climates from a common underlying species response. This adaptation occurs in the initial depth of primary dormancy and the subsequent balance of induction and relief so that cycling behaviour may differ within and between ecotypes if the environment changes.

Initial depth of dormancy is determined by both genetics and environmental exposure pre- and post-shedding (Finch-Savage and Footitt, 2017). The effect of the latter is illustrated here in data from seeds of the winter annual ecotype Cvi from the same harvest, but with different depths of dormancy resulting from post-harvest conditions. Seeds with greater depth of dormancy (Fig. 1) did not become light sensitive upon exposure to low temperature, but became more dormant. Whereas, a proportion of seeds in a less dormant seed lot (Fig. 2) became light sensitive before the whole seed population subsequently

415 became more dormant on continued exposure. Cycling behaviour therefore differed
416 depending on the environmentally determined initial depth of dormancy. In both seed lots
417 dormancy in Cvi was then relieved by exposure to higher temperatures (Footitt *et al*. 2011;
418 Huang *et al*. 2015). This is apparently in direct contrast to the results with the summer
419 annual ecotypes Bur, Col-0 and Ler in which increasing temperature accelerated the
420 induction of secondary dormancy (Figure 3; Cone and Spruit, 1983; Huang *et al.* 2015,
421 Springthorpe and Penfield, 2015). However, Col-0 can behave as both a winter and summer
422 annual in the field (Springthorpe and Penfield, 2015) suggesting secondary dormancy may
423 also be relieved by high temperature as seen in Cvi depending on the environment before
424 and after shedding. Thus in Col-0, further induction of secondary dormancy by low
425 temperature may be required before a change to high temperature results in relief.
426 However, this intriguing aspect of dormancy cycling in Col-0 is yet to be demonstrated.
427
428 The above results raise the question of how dormancy cycling is driven by temperature and
429 time (thermal time) to alter the balance between induction and relief of dormancy as part of
430 a dormancy continuum. In this continuum, as primary dormancy in the dispersed seed is
431 relieved, in response to the prevailing environmental conditions (predominantly
432 temperature), the same conditions will start to induce secondary dormancy if the
433 environmental signals required to remove the final layer of dormancy are not received. This
434 behaviour is consistent with the hypothesis that temperature impacts the rate of dormancy
435 induction and relief independently, but importantly that these processes may occur
436 simultaneously (Totterdell and Roberts, 1979; Battla *et al*. 2009). These opposing processes
437 are largely governed by the environmental sensitivity of the ABA/GA hormone balance
438 (Finch-Savage and Leuber-Metzger, 20016; Finch-Savage and Footitt, 2017). Initial primary

dormancy level determines the temperature sensitivity of both induction and relief via changes in this balance. The hypothesis implies that the terms primary and secondary dormancy are only descriptive of sequences in the cycle with no physiological relevance as dormancy is a continuum, and only the level changes.

The protocol presented could be used to test this hypothesis and further develop our understanding of dormancy cycling by evaluating responses of different ecotypes to temperature and water potential. The ecotypes used in the present work included Col-0 as the common genetic background for 1,000's of mutant lines (http://arabidopsis.info/) to facilitate genetic dissection of dormancy cycling. We discuss below how the protocol also has great potential as an investigative tool in advancing our understanding of the role of genes in dormancy regulation.

Regulation of dormancy cycling.

How ABA and GA -signalling pathways are co-ordinated during dormancy cycling by temperature and water potential is not fully understood (Finch-Savage and Footitt, 2017). It was argued previously (Footitt *et al.* 2013) that changing temporal signals linked to the transcription of *DOG1*, *MFT*, *PHYA* and *CIPK23* drives regulation of dormancy cycling. *DOG1* and *MFT* expression contributes to thermal time sensing linked to changes in *CIPK23* and *PHYA* expression that result in altered sensitivity to spatial signals (nitrate and light respectively) indicating suitability for germination. The data presented for mutants of these genes subjected to the laboratory simulation of dormancy cycling (Fig. 3) supports the correlative observations made in the field. Thermal time analysis showed that dormancy induction in the absence of DOG1 (*dog1-2*) was linear with thermal time and exponential in

its presence (Fig. 5). This adds to the contention that DOG1 is part of a thermal mechanism sensing an annual seasonal pattern (circannual rhythm) and may amplify thermal signals by increasing ABA sensitivity. Mutants in *CIPK23* and *PHYA* show an increased induction of secondary dormancy. Both PHYA and CIPK23 also influence hormone signalling consistent with the importance of the dynamic ABA/GA balance determining dormancy levels in response to environmental signals (Finch-Savage and Footitt, 2017).

DOG1 and MFT: During seed development *DOG1* is absolutely required for the induction of dormancy (Dekkers *et al.* 2016). However, in *dog1-1* (Ler background) low dark germination was seen in fresh seeds that could be removed by low temperature indicating a low level of primary dormancy was present at maturity (Bentsink *et al.* 2006). In the dormancy simulation, high temperature alone did not induce secondary dormancy in *dog1-2* as it had high levels of dark germination followed by full germination on transfer to light (Fig. S5). However, cold preconditioning at -1 MPa induced a low level of secondary dormancy at the end of the initial cold phase. Light was increasingly unable to remove the final layer of dormancy in Col-0 but not *dog1-2* (Fig. 2(a) days 21 to 28). This small loss of sensitivity to light indicates that in the Col-0 genetic background secondary dormancy induction was starting to dominate its relief. On transfer to the higher temperature this level of secondary dormancy was sufficient to prevent dark germination in *dog1-2* as well as in Col-0 while dormancy induction increased to the point where seeds were no longer light sensitive (Fig. 3). On the basis that any environmental signal that widens the conditions required for germination is in effect altering dormancy (Finch-Savage and Footitt, 2012) we conclude that the induction of a light requirement and the decreasing sensitivity to light with increasing thermal time is evidence for the induction of secondary dormancy in *dog1-2*. This

indicates that these conditions allow other factors to impose secondary dormancy in the absence of *DOG1.* One potential candidate is *MFT*.

Induction of secondary dormancy in thermal time was slower in *dog1-2* than in *mft2* indicating the primacy of *DOG1* over *MFT* during dormancy induction. Further research is required to confirm a role for *MFT* in thermal sensing. The greatly reduced induction of dormancy in *dog1-2* is consistent with DOG1 amplifying thermal signals via increased sensitivity to ABA. The dramatically lower ABA sensitivity of *dog1-2* reported here supports this (Fig. 6(a-c)).

The loss of ABA sensitivity in *mft2* shows MFT contributes positively to ABA signalling (Fig. 6). This is via the oxylipin, 12-oxo-phytodienoic acid (OPDA), which acts through MFT to induce ABA biosynthesis and sensitivity (Dave *et al.* 2016). Then MFT and ABA via a feedback loop enhance OPDA levels further contributing to DOG1 germination repression (Dave *et al.* 2016) explaining the ABA hypersensitive germination of *MFT* overexpressing lines (Hu *et al.* 2016). The delayed response to ABA compared to *dog1-2* may reflect declining ABA levels when the OPDA pathway is blocked. In contrast, fully after-ripened *mft2* seeds are ABA hypersensitive (Xi *et al.* 2010). This may reflect a changing temporal sensing role for MFT dependent on ecotype and the seasonal onset of the dormancy cycle as reflected in altered timing of *MFT* transcription in the field (Footitt *et al.* 2013, 2014). This role for *MFT* in shallow dormancy when *DOG1* levels are low is discussed elsewhere (Finch-Savage and Footitt, 2017).

510 During the final low temperature phase secondary dormancy is broken faster in *dog1-2* and
511 *mft2*, than in the wild type. Low temperature treatment then re-induced secondary
512 dormancy in the wild type but not in these mutants showing that dormancy cycling at low
513 temperature is compromised.
514
515 **PHYA and CIPK23**: Unlike *mft2* and *dog1-2* secondary dormancy was induced in *phyA and*
516 *cipk23* by low temperature, which then accelerated on transfer to higher temperatures (Fig.
517 3(a) & S2). This induction of secondary dormancy at high temperature, its relief and re-
518 induction in the second low temperature phase is consistent with increased ABA sensitivity
519 compared to Col-0. This is supported by the ABA hypersensitivity of *cipk23* (Fig. 5(a-c)). The
520 limited ABA response of *phyA* reflects the increased contribution of other negative
521 regulators of germination potential in this mutant (Ibarra *et al.* 2013).
522
523 **PHYA:** PHYA is responsible for the Very Low Fluence Response whereby the final layer of
524 dormancy is removed by brief exposure to light during soil disturbance (Battla and Benech-
525 Arnold, 2014). The increased sensitivity of *phyA* seeds to temperature and water stress is
526 consistent with enhanced ABA sensitivity (Fig 3a and Fig 6(a-c)). Transcriptome comparisons
527 between wildtype and *phyA* seeds support this with 11% of the expressed transcriptome
528 significantly regulated by PHYA (Ibarra *et al.* 2013). Of those significantly up regulated by
529 PHYA, 7% are transcription factors linked with auxin and GA responses; and ABA catabolism.
530 While down regulated genes contain representatives of the ABA signalling pathways and
531 DELLA genes that relieve repression of GA signalling (Ibarra *et al.* 2013). So in *phyA* seeds
532 the balance of the ABA/GA signalling pathways favours ABA amplifying the response to
533 dormancy inducing temporal signals.

534

535 **CIPK23:** This protein forms a calcium sensing complex with CBL1 or CBL9 (CALCINEURIN B-
536 LIKE PROTEIN), which is involved in iron, nitrate and potassium transport and sensing (Leran
537 *et al.* 2015; Manik *et al.* 2015; Tian *et al.* 2016). Its role in regulating nitrate transport and
538 signalling by the NITRATE TRANSPORTER1.1 (NRT1.1) transceptor (duel nutrient
539 transport/signalling function) and the crucial role this plays in the regulation of ABA levels in
540 seeds is well characterised (reviewed in Finch-Savage and Footitt, 2017).

541

542 The ABA hypersensitivity of *cipk23* seeds (Fig 6(a-c)) indicates ABA signalling is enhanced in
543 the absence of CIPK23. In the field, low dormancy is coincident with increased nitrate
544 sensing, which is preceded by enhanced NRT1.1 expression and reduced CIPK23 expression.
545 The subsequent onset of secondary dormancy induction appears to reduce nitrate signalling
546 below threshold levels both by reducing the amount of NRT1.1 and its phosphorylation via
547 CIPK23-CBL1/9 (reviewed in Finch-Savage and Footitt, 2017; Footitt *et al.* 2011; 2013, 2014).
548 Here Col-0 and *cipk23* lose light but not nitrate sensitivity during induction of secondary
549 dormancy suggesting loss of nitrate sensitivity is related to NRT1.1 protein levels and the
550 action of factors such as DOG1 that regulate deep dormancy (reviewed in Finch-Savage and
551 Footitt, 2017). CIPK23-CBL complexes also have other functions, for example as nutrient
552 sensors to monitor mineral homeostasis in general (Tian *et al.* 2016). Further work is
553 therefore needed to fully understand the role of CIPK23 in dormancy regulation.

554

555 **Dormancy regulation and clock genes:**

556 The annual seasonal rhythm of soil temperature (Fig. 6a and b) was correlated with
557 transcriptional responses of the dormancy related genes discussed above (see Table S1).

558 During seasonal bud dormancy regulation in trees, perception of seasonal temperature
559 signals involves components of the circadian clock (Cooke *et al.* 2012). We tested if this
560 could also be occurring in seeds by analysis of clock mutants in the laboratory simulation
561 and by measuring clock gene expression over an annual cycle in the field. The results
562 obtained were consistent with the balance between the evening and morning phases of the
563 clock contributing to the interpretation of temperature signals (thermal time) that
564 determine cycles of dormancy induction and relief.
565
566 **Clock mutants in the laboratory simulation:** In this series of laboratory simulations
567 successive relatively long-term incubations at constant temperatures in the dark show the
568 clock has an impact on dormancy status without an imposed external daily rhythm (Fig. 3,
569 S2, S3, & S4). In the parental wild type (Col-0) secondary dormancy was induced on transfer
570 to high temperature and increased further as temperature was raised (20 > 25 > 30°C). Lines
571 with mutations in the morning genes *LHY* and *CCA1* (*lhy20 cca1-1* and *lhy20 cca1-1 toc1-2*)
572 had the highest ABA sensitivity and the most rapid induction of secondary dormancy.
573 Whereas, the triple mutant *prr5 11 prr7-11 prr9-10* had the lowest ABA sensitivity and
574 slowest induction (Fig. 6 (d-f)). This disruption of the morning loop by mutations in *LHY* and
575 *CCA1* would reduce repression of the evening loop genes *TOC1*, *Gi* and the evening complex
576 genes *LUX*, *ELF3* and *ELF4* (Pokhiloko *et al.* 2013). The *prr5 11 prr7-11 prr9-10* mutant would
577 reduce repression of *LHY* and *CCA1*. Therefore, this result indicates a critical balance
578 between the morning and evening signalling components of the clock influences the
579 induction of dormancy. It further implies that in the absence of a fully functioning morning
580 loop repression of *TOC1, Gi* and the evening complex genes is incomplete. This is consistent
581 with observations of delayed bud burst (loss of dormancy) in *Populus* LHY mutants (Ibanez

582 *et al.* 2010). These data are also consistent with clock gene transcription recorded during
583 the annual soil temperature cycle in the field (Fig. 4) and are discussed below. This field data
584 indicates the annual seasonal cycle is analogous to an extended diurnal cycle with low
585 winter temperatures representing the evening phase, and summer temperatures the
586 morning phase (circannual dormancy rhythm). Thermal time analysis (Fig. 5) shows that
587 dormancy cycling responds to the strength of the inductive thermal time signal generated
588 by the clock.
589
590 **Annual clock gene expression in the field:** We followed gene expression in the contrasting
591 ecotypes Bur (summer annual) and Cvi (winter annual).The transcript profiles of evening
592 genes increased with falling temperature and therefore in general were negatively
593 correlated to the annual soil temperature cycle in both ecotypes (Table S1). Surprisingly, the
594 morning genes *LHY* (in Cvi) and *CCA1* (in Bur) have the same transcript profiles as *TOC1*.
595 While only *LHY* transcription in Bur correlates positively with temperature. This contrasts
596 the general situation in the clock where the transcript profile of *TOC1* is in the opposite
597 phase to both *LHY* and *CCA1* (Salome and McClung, 2005; Gould *et al.* 2006). However, it is
598 consistent with high transcription of *TOC1* and *LHY* in Chestnut internodes during winter
599 when the clock becomes arrhythmic (Ibanez *et al.* 2008). Notably *LHY* (in Cvi) and *CCA1* (in
600 Bur) transcription do not return to the opposite phase of *TOC1* in the warm summer
601 months. It is also notable that in Bur *TOC1* transcription also increases with summer
602 temperature and at that point is similar to both *LHY* and *CCA1*. This suggests that adaptation
603 of dormancy cycling to the environment may involve allelic variation in clock genes as seen
604 in Drosophila (Yamada and Yamamoto, 2011).
605

Components of the clock will alter the central integrating ABA/GA balance controling dormancy cycling. *TOC1* and the clock are involved in the gating of ABA responses (Seung *et al.* 2012). *TOC1* is induced by ABA and interacts with genes involved in ABA signalling responses (Seung *et al.* 2012). In addition it interacts with the positive regulator of dormancy *ABA INSENSITIVE3* (*ABI3*) (Kurup *et al.* 2000). ABI3 mutants also exhibit altered circadian rhythms (Pearce, 2003). The consequence of increased *TOC1* transcription therefore appears to be an upregulation in ABA signalling. Gibberrelin biosynthesis is repressed by the evening loop with increased expression of the GA biosynthesis gene *GA20OX2* found in *toc1*; and increased levels of bioactive Gibberrelins and *GA20OX2* found in *elf3* (Atamian and Harmer, 2016). This again indicates evening loop involvement in dormancy cycling.

Dormancy and ABA levels initially increase together, but a point is reached were dormancy increases are ascribed to increasing ABA sensitivity via DOG1 (Footitt *et al.* 2011). Interestingly circadian rhythm micro array data from Col-0 seedlings (Edwards and Millar 2007) shows rhythmic *DOG1* transcription (See: http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi?dataSource=Light_Series) (Fig. S10). In contrast, morning gene transcription is more positively correlated with genes up-regulated in the spatial sensing phase of the dormancy cycle (Table S1).

Circannual dormancy rhythm:

Annual cycling of the depth of dormancy is well documented (Baskin and Baskin, 1998) and understanding of how this is regulated by a range of dormancy mechanisms in response to environmental signals is developing (Finch-Savage and Footitt, 2017). These mechanisms

operate via a central integrating ABA/GA balance to time germination completion in the optimum season and habitat. Here we confirm the key involvement of *DOG1*, *MFT*, *CIPK23* and *PHYA* in regulating the depth of dormancy. Furthermore we show based on mutant analyses and transcript profiles that the balance between the evening and morning phases of the clock also reflects this circannual dormancy rhythm. Based on the thermal time and ABA sensitivity data, dormancy cycling appears to respond to the strength of the inductive thermal time signal generated by the clock. Further directed research is required to test these hypotheses and provide detail of the clocks involvement. Nevertheless, circannual rhythms for germination timing are seen in seeds of the desert annual *Mesembryanthemum nodiflorum* and in cysts of the marine dinaflagellate *Alexandrium* in constant conditions over several years (Gutterman and Gender 2005; Matrai *et al.* 2015) and may be part of a bet hedging strategy. How a circannual clock contributes to and maintains annual rhythms over several years is unclear.

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Supporting information

Text S1. Materials and methods

Table S1. Correlation table

Table S2. Primers

Figure S1. Box layout for incubation of seeds at reduced water potential.

Figure S2. Simulated dormancy cycling in dormancy related mutants at 25°C and 30°C.

Figure S3. Simulated dormancy cycling in clock mutants at 20°C, 25°C and 30°C.

Figure S4. Simulated dormancy cycling in *CCA1* and *LHY* overexpressing lines at 20°C, 25°C and 30°C.

Figure S5. Response of dormancy related mutants when placed directly in high temperature without cold conditioning at low water potential.

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840 **Figure S6. Thermal time analysis of dormancy induction at high temperature following low**

841 **temperature conditioning of the dormancy mutants' *dog1-2* and *mft2* and clock mutants.**

842

843 **Figure S7. ABA sensitivity of Col-0 and Ler wild types and *CCA1* and *LHY* overexpressing**

844 **lines.**

845

846 **Figure S8. Dark germination of Col-0, and the dormancy mutants *dog1-2* and *mft2*.**

847

848 **Figure S9. Dark germination of Col-0, clock mutants and *CCA1* and *LHY* overexpressing**

849 **lines.**

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851 **Figure S10. *DOG1* transcript level in Col-0 seedlings entrained to a light/dark cycle.**

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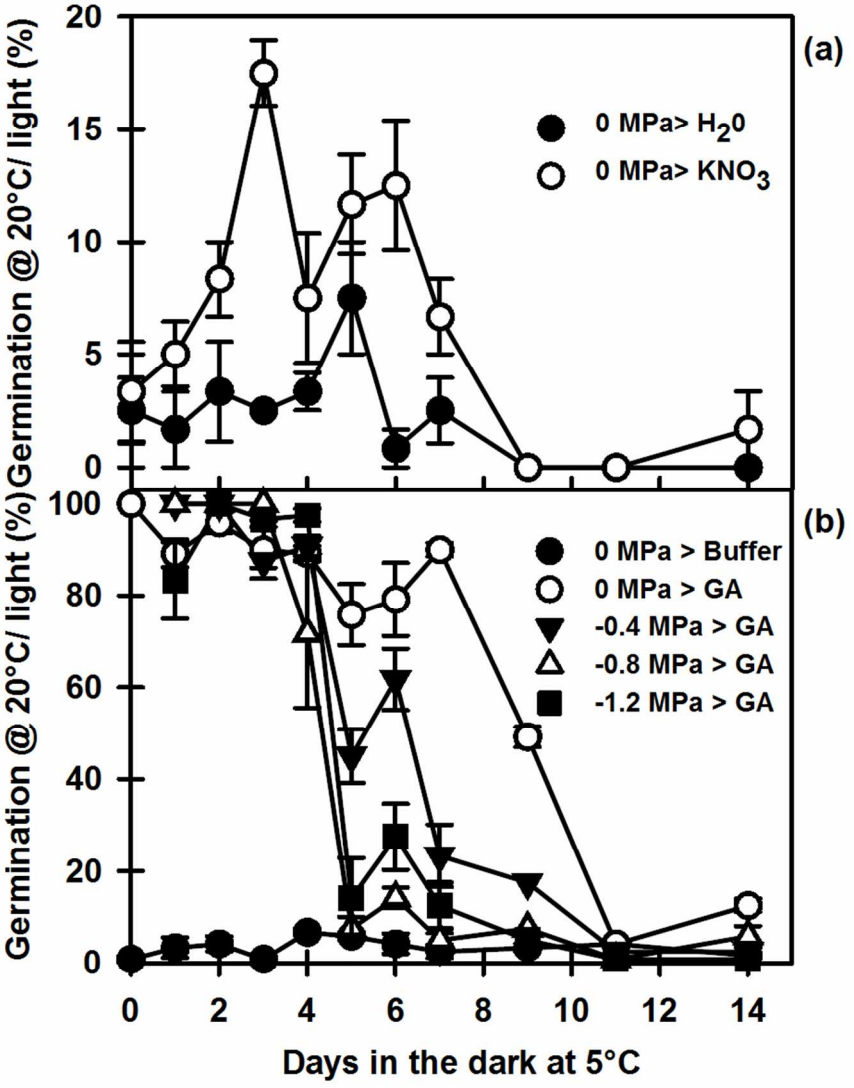

Figure 1. Induction of secondary dormancy in Cvi in response to cold stratification and decreasing water potential. Primary dormant Cvi seeds were incubated at 5°C/dark on water or a range of water potentials from -0.4 to -1.2 MPa. At increasing periods of time dormancy status was determined by measuring germination following transfer of seeds to (a) water or 10 mM KNO3, or (b) a buffer control or 250 µM GA4+7 buffered at pH 5.0 at 20°C/light for 28 days. Data are mean ± SE (n = 3). Absence of error bars indicates SE is smaller than the symbol.

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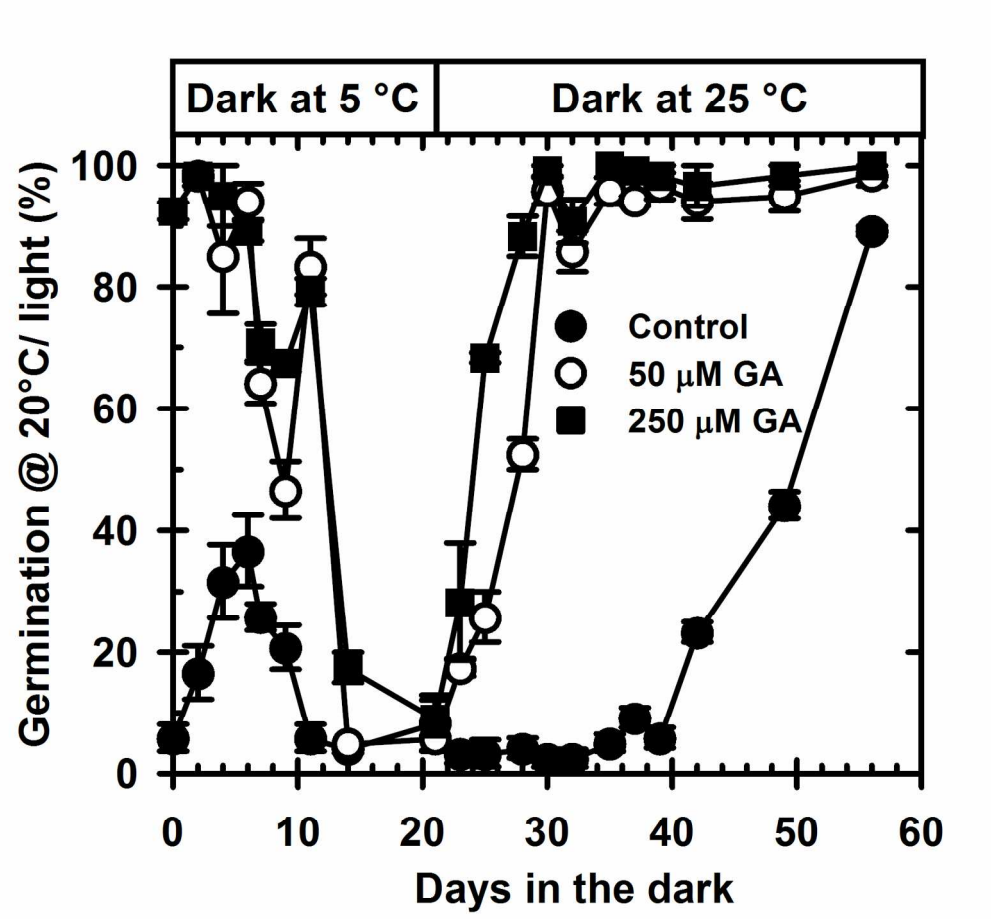

Figure 2. Simulated dormancy cycling in Cvi: Seeds were incubated at 5°C/dark at -1.2 MPa for up to 21 days before being transferred to water at 25°C/dark. At increasing periods of time dormancy status was determined by measuring germination following transfer of seeds to a buffer control, 50 or 250 μM GA4+7 buffered at pH 5.0 at 20°C/light for 28 days. Data are mean ± SE (n = 3). Absence of error bars indicates SE is smaller than the symbol.

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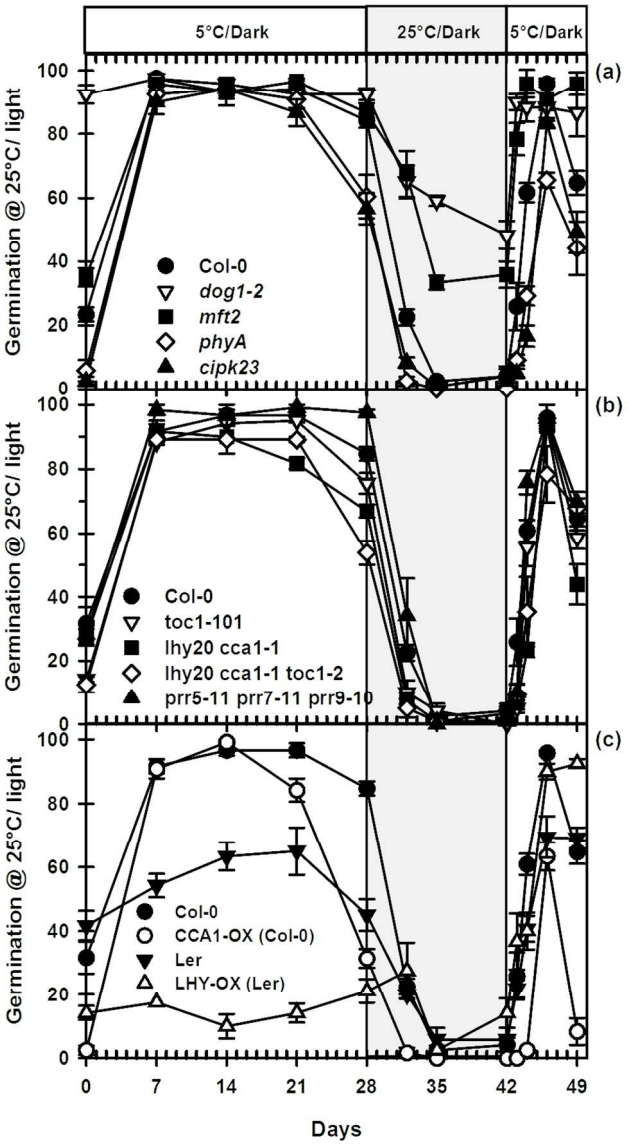

Figure 3. Simulated dormancy cycling in Col-0, Ler and mutants in dormancy related and clock genes. Following 5°C/dark at -1.0 MPa for 28 days seeds were transferred to water and incubated in the dark at 25°C for 14 days before transferring to 5°C/dark. At increasing intervals dormancy status was determined by measuring germination on water at 25°C/light for 14 days. (a) Dormancy related mutants. (b) Circadian clock mutants. (c) CCA1 and LHY overexpressing lines. Data are mean ± SE (n = 3). Absence of error bars indicates SE is smaller than the symbol.

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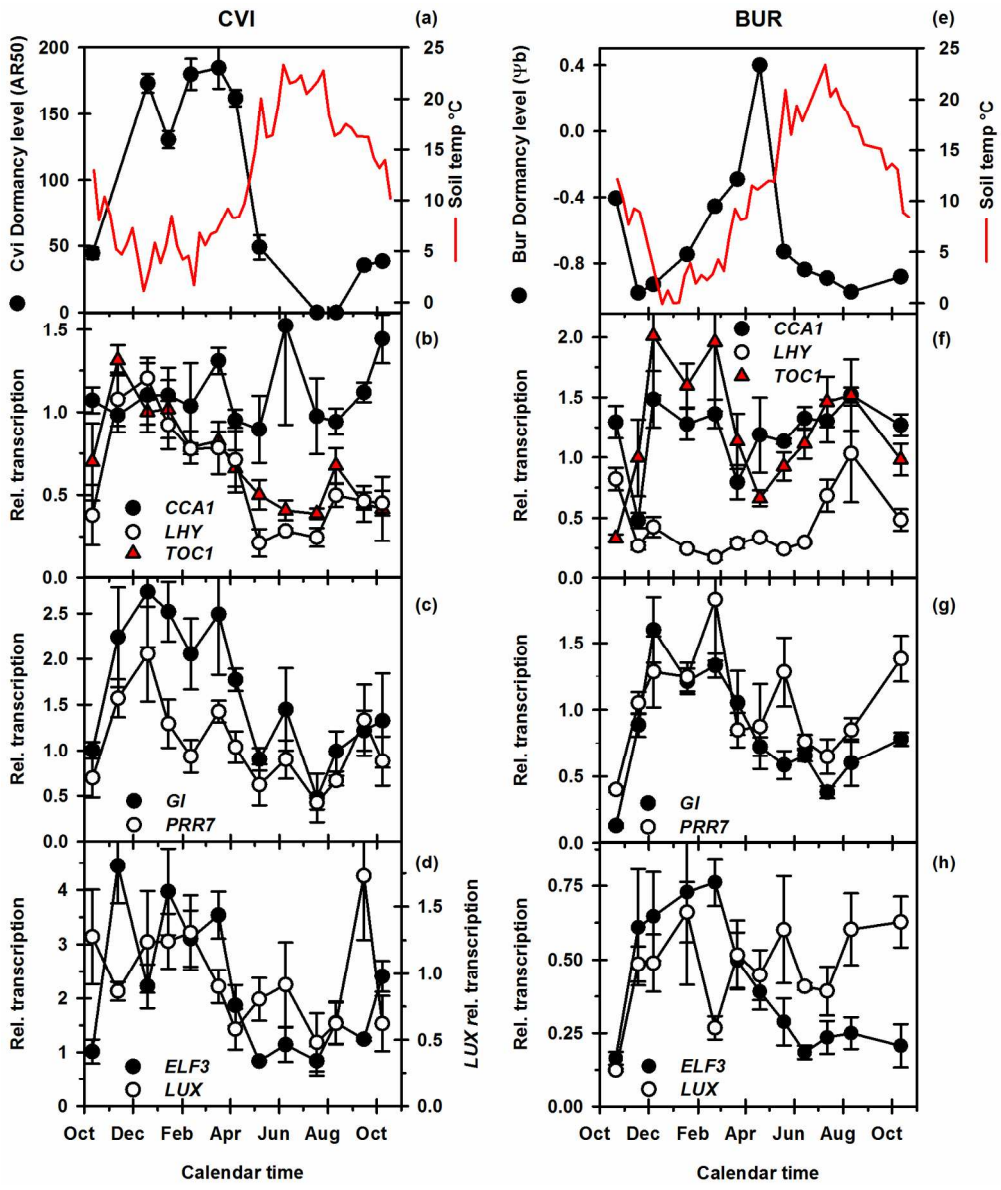

Figure 4. Seasonal coordination of clock gene transcription in winter (Cvi) and summer annual (Bur) ecotypes. Depth of dormancy in (a) Cvi (time to 50% after-ripening (AR50)) and (e) Bur (base water potential (Ψb)) with soil temperature at seed depth (Data from Footitt et al. 2011 and 2013). Transcription profiles of the morning genes CCA1, LHY, and the evening gene TOC1 in (b) Cvi and (f) Bur. Transcription profiles of GI and PRR7 in (c) Cvi and (g) Bur. Transcription profiles of evening complex genes ELF3 and LUX in (d) Cvi and (h) Bur.

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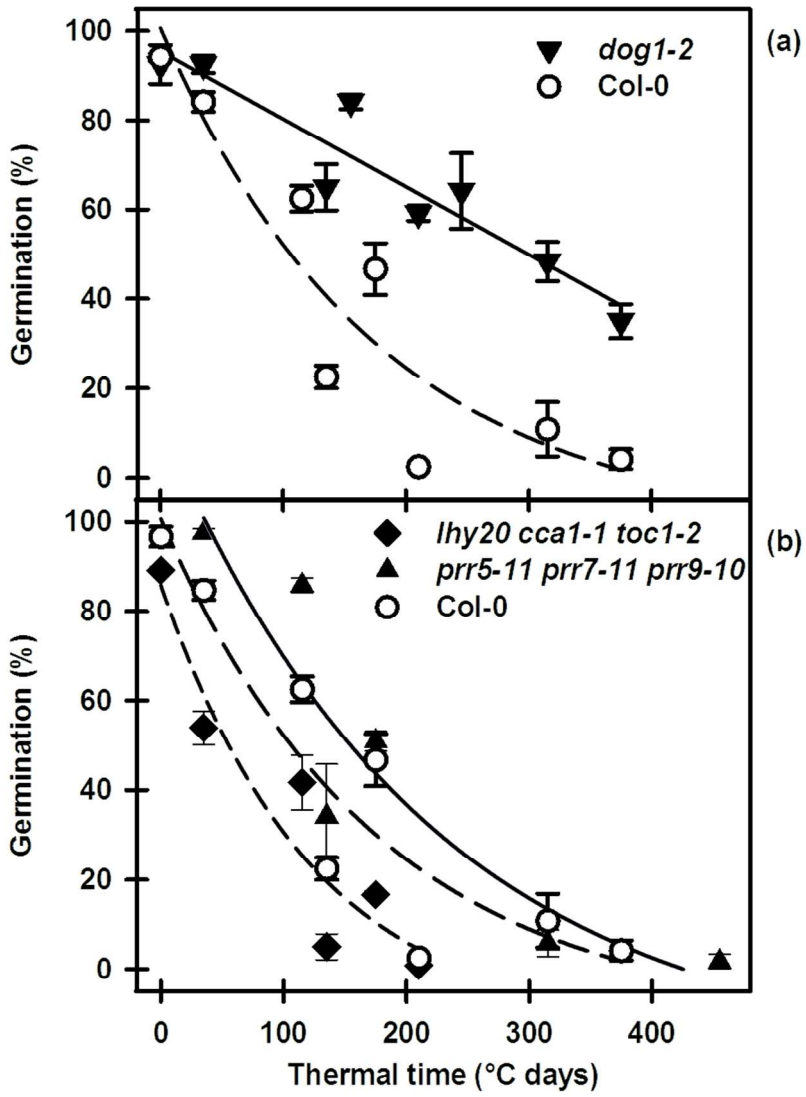

Figure 5. Thermal time analysis of dormancy induction at high temperature following low temperature conditioning. (a) Wild type (Col-0) and the dormancy mutant, dog1-2. (b) Wild type (Col-0) and the circadian clock mutants, lhy20 cca1-1 toc1-2 and prr5-11 prr7-11 prr9-10. Data from Fig. 3, S1 and S2 are replotted against thermal time (sum of temperature above 0°C) for secondary dormancy induction at 20, 25 and 30°C. The response to thermal time fits the following relationships: Exponential decay (3 parameter) regressions describe Col-0 (R2= 0.972), lhy20 cca1-1 toc1-2 (R2=0.897) and prr5-11 prr7-11 prr9-10 (R2=0.860); while a linear regression describes dog1-2 (R=0.928). The same data for Col-0 appears in (a) and (b).

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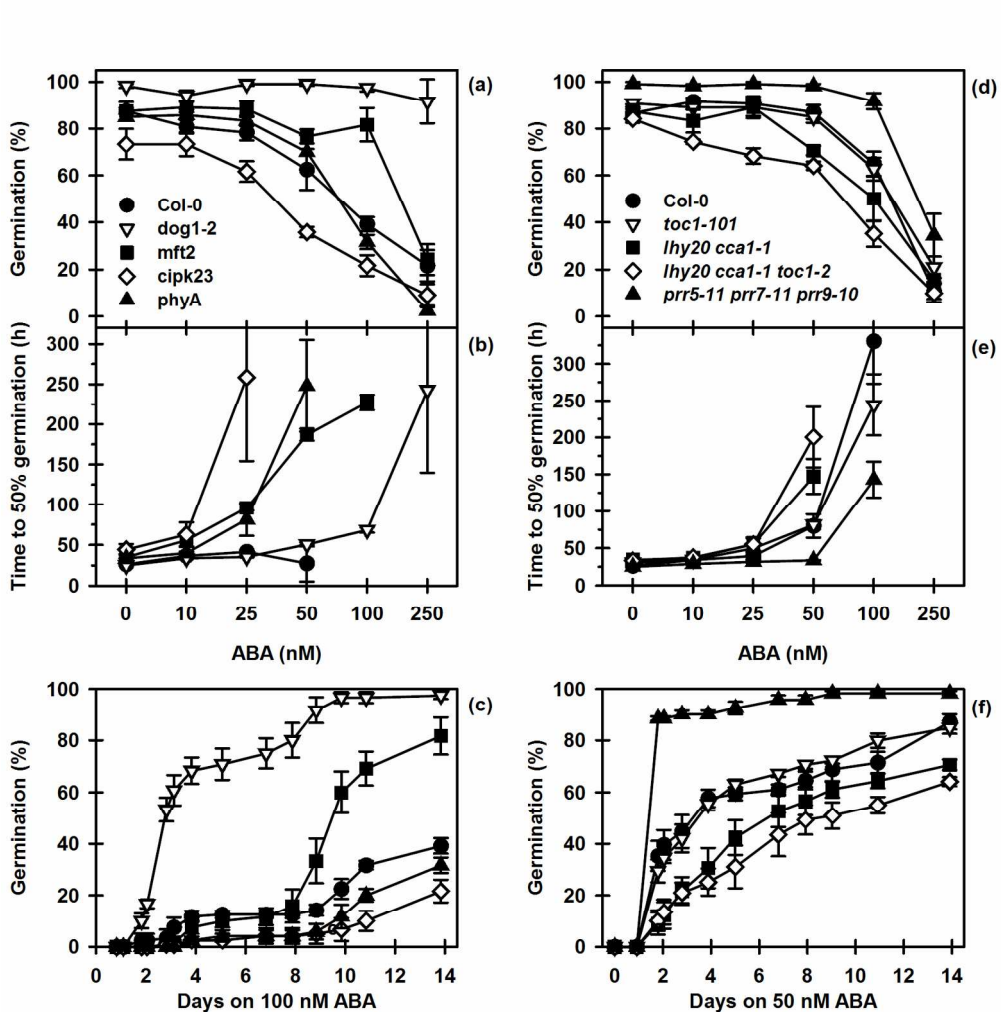

Figure 6. ABA sensitivity of dormancy and clock mutants. Following three days at 5°C/dark on water seeds were transferred to ABA (10 – 250 nM) in buffer at pH 5.0 and cumulative germination recorded during incubation at 25°C/light over 14 days. Final germination at each concentration after 14 days (a) and (d). The time to 50% germination (b) and (e) in hours (h) of data in (a) and (d) respectively. Cumulative germination of dormancy mutants in the presence of 100 nM ABA (c). Cumulative germination of clock mutants in the presence of 50 nM ABA (f). Data are mean ± SE (n = 3). Absence of error bars indicates SE is smaller than the symbol.

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